Changes in Expression of Ia, Thy-1, and Ly-5 Antigens in Epidermal Cells During Delayed Contact Sensitivity Reactions in Mice: Flow Cytometric Analysis*

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Ia antigen-bearing (Ia^+) Langerhans cells have attained an important position as immunocompetent cells in the epidermis. Recently there have been successive reports on other new possible candidates for immunocompetent cells in the epidermis, i.e., Ia^+ keratinocytes and dendritic Thy-1 antigen-bearing ($Thy\text{-}1^+$) epidermal cells which also express Ly-5 antigen and asialo-GM_1. Based on our previous findings that in allergic contact sensitivity reactions, keratinocytes express Ia antigen 3–9 days postchallenge, in this report, we have attempted to define more clearly the dynamic changes of Ia^+ keratinocytes and dendritic $Thy\text{-}1^+$ epidermal cells by enumeration of the precise percentages of Ia^+, $Thy\text{-}1^+$, and Ly-5 antigen-bearing ($Ly\text{-}5^+$) cells in epidermal cells at various times of the challenge phase in allergic contact sensitivity reactions by use of a fluorescence-activated cell sorter. By 24 h postchallenge, the percentages of Ia^+, $Thy\text{-}1^+$, and $Ly\text{-}5^+$ cells showed hardly any change. There were approximately 2% Ia^+ cells, 50% $Thy\text{-}1^+$ cells which consist of 2 populations (i.e., 45% weakly Thy-1 antigen-positive cells and 4% strongly Thy-1 antigen-positive cells), and 3.5% $Ly\text{-}5^+$ cells. From 48 h postchallenge, however, the percentage of Ia^+ cells and that of $Thy\text{-}1^+$ cells began to increase and reached a plateau, with approximately 20% Ia^+ cells and 70% $Thy\text{-}1^+$ cells, respectively, at 120 h postchallenge. The change of the percentages of $Ly\text{-}5^+$ cells appears to correspond to that of strongly Thy-1 antigen-positive cells. Only at 48 h postchallenge, $Ly\text{-}5^+$ cells and strongly Thy-1 antigen-positive cells showed a small increase in number, comprising approximately 10% of the epidermal cells. These data suggest that among $Thy\text{-}1^+$ epidermal cells, strongly Thy-1 antigen-positive cells correspond to dendritic $Thy\text{-}1^+$ epidermal cells, and in contact sensitivity reactions in mice, dendritic $Thy\text{-}1^+$ epidermal cells show only a minor dynamic change in contrast to Ia^+ cells, in which more than 15% of keratinocytes express Ia antigen from 48 h postchallenge. *J Invest Dermatol 86:121–124, 1986*

In the normal epidermis, the expression of Ia antigen is limited to Langerhans cells [1]. However, the Ia antigen expression has been recognized on the keratinocyte surface in several skin disorders [2–6]. We demonstrated that this phenomenon is frequently observed in the dermatoses characterized by lymphocytic exocytosis into the epidermis [7]. Experimental induction of Ia antigen on the keratinocyte in vivo was reported in graft-vs-host disease [8–11] and allergic contact dermatitis in mice, in which Ia antigen was recognized on the keratinocyte 3–9 days postchallenge [12].

Recently a new dendritic epidermal cell type of bone marrow origin has been reported other than Langerhans cell [13,14]. This cell is Thy-1 alloantigen-positive, Ia antigen-negative [13,14], Ly-5 leukocyte common antigen-positive [13], and asialo-GM_1-positive [15]. However its definite functional characterization still is not clear. The first purpose of this study was to obtain precise information about how many keratinocytes express Ia antigen in the challenge phase of allergic contact sensitivity reactions. Another purpose was to clarify whether the dendritic $Thy\text{-}1^+$ epidermal cells play any role in allergic contact sensitivity reactions, which show a dramatic change in number.

MATERIALS AND METHODS

Animals Male C3H/HeJ($H\text{-}2^k$) mice, obtained from Funabashi Farm, Chiba, Japan, were 9–12 weeks old when used.

Reagents 2,4,6-Trinitrochlorobenzene (TNCB) was purchased from Tokyo Kasei Ltd., Tokyo, Japan, and protected from light during storage. Other materials used were obtained from the following sources: bovine serum albumin and crude DNase from Sigma Chemical Company, St. Louis, Missouri; Eagle's minimum essential medium (MEM) and fetal bovine serum (FBS) from Gibco Laboratories Inc., Grand Island, New York; crude trypsin from Difco Laboratories, Detroit, Michigan; fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ of goat antimouse immunoglobulins from Tago Inc., Burlingame, California; monoclonal anti-Ia^k(2) antibody and FITC-conjugated monoclonal anti-Thy 1.2 antibody from Becton Dickinson, Sunnyvale, California; monoclonal anti-Ly-5.1 antibody from New England Nuclear, Boston, Massachusetts.

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Abbreviations:
FACS: fluorescence-activated cell sorter
FBS: fetal bovine serum
FITC: fluorescein isothiocyanate
Ia^+: Ia antigen-bearing
$Ly\text{-}5^+$: Ly-5 antigen-bearing
MEM: minimum essential medium
PBS: phosphate-buffered saline
$Thy\text{-}1^+$: Thy-1 antigen-bearing
TNCB: 2,4,6-trinitrochlorobenzene

Sensitization and Elicitation of Contact Sensitivity Contact sensitization was assessed as previously described [16]. Briefly, mice were painted with 100 μl of 7% TNCB (4:1, acetone:olive oil) on the shaved abdomen on day 0 and bilateral ears were challenged with 100 μl of 1% TNCB in olive oil on day 6. At various times after elicitation, groups of 5 mice were sacrificed, and the thickness of the ears was measured using an engineer's micrometer (Mitutoyo, Tokyo, Japan). Data are expressed as increment in ear thickness with standard error.

Epidermal Cell Suspensions At various times after elicitation, single epidermal cell suspensions were prepared from the ears of mice by the method of Steinmuller and Wunderlich [17]. Briefly, after depilation and disinfection, the ears were divided into 2 sheets between the ventral and dorsal side. These sheets were washed in 0.02% EDTA in phosphate-buffered saline (PBS) and immersed in 0.5% crude trypsin in PBS for 2 h at 37°C. The incubation time in trypsin was longer than 60–80 min of the original method [17] because we needed to obtain the whole epidermal cells including the basal layer and upper epidermis at different times after challenge; we confirmed that the prolongation of trypsin treatment had no influence on the obtained results in our previous study. Subsequently, the epidermal portions were manually dissected from the dermis. They were further treated with 0.025% crude DNase in PBS for 20 min at 37°C and then epidermal cell suspension was performed by vigorous pipetting and filtration through a 60-mesh wire sieve to remove cellular aggregates and sheets of fully keratinized cells. This single cell suspension was washed twice with MEM supplemented with 10% heat-inactivated FBS.

Analysis of Epidermal Cell Suspensions by Fluorescence-Activated Cell Sorter (FACS) The epidermal cells from fresh suspensions were resuspended in PBS at a concentration of 20×10^6 epidermal cells per ml. One hundred microliters of the cell suspension were incubated with 150 μl of monoclonal antibodies anti-Ia^k, anti-Ly-5.1, and FITC-conjugated anti-Thy 1.2 at a concentration determined to give optimal staining or of the control unreactive mouse monoclonal antibody for 45 min at 4°C, and then the cells were washed 3 times in PBS. The cells treated with anti-Ia^k antibody and anti-Ly-5.1 antibody were further incubated with the $F(ab')_2$ fraction of FITC-conjugated goat antimouse immunoglobulins at a 1:20 dilution for 30 min at 4°C and washed 3 times in PBS. After the final wash, the suspension was filtered through gauze to remove any cell aggregates. Ten thousand cells per assay were analyzed by passage through a FACS analyzer (Becton-Dickinson). A fluorescence histogram was obtained for each sample, with fluorescence intensity (log units) being expressed on the X-axis and cell number on the Y-axis.

RESULTS

Time Course of Ear Swelling After Challenge Groups of 5 mice were sacrificed at various times after challenge and their ear swelling was measured. A peak response was observed at 48 h after challenge (Fig 1).

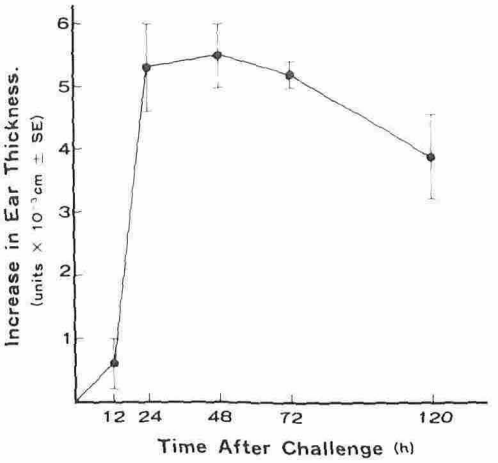

Figure 1. Time course of ear swelling. C3H mice were sensitized with 100 μl of 7% TNCB (4:1 acetone:olive oil) and 6 days later their ears were challenged with 1% TNCB in olive oil. Ear swelling was measured at various times after challenge. *Vertical bars* indicate mean ± SD.

Kinetic Analysis of Pattern in Cytofluorograph After Challenge Cytofluorographs with anti-Ia^k, anti-Thy-1, and anti-Ly-5 monoclonal antibodies were obtained from the epidermal cells of ears of mice at various times after challenge (Fig 2). A dynamic change was observed only in cytofluorographs with anti-Ia^k antibody. Namely, the percentage of Ia^+ cells in the epidermal cells suddenly rose from 48 h postchallenge and showed a gradual increase until 120 h postchallenge. Thy-1^+ cells began to increase in number from 12 h postchallenge, making a plateau at 48 h postchallenge. By contrast, in the cytofluorographs with anti-Ly-5 antibody, only a small change was recognized at 48 h postchallenge. Table I showed the precise percentages of the epidermal cells reactive with these 3 monoclonal antibodies at various times after challenge. In Table I, 2 different percentages are shown in regard to Thy-1^+ cells. The cytofluorograph with anti-Thy-1 antibody showed 2 peaks at 0 h postchallenge (Fig 2), i.e., a broad peak with low fluorescence intensity and a small one with high fluorescence intensity, suggesting that these cells consist of 2 different cell populations. Thus we divided these cells into weakly Thy-1 antigen-positive cells and strongly Thy-1 antigen-positive cells. The increase in Thy-1^+ cells recognized after 12 h postchallenge was due to that in weakly Thy-1 antigen-positive cells, whereas the strongly Thy-1 antigen-positive cells showed a small increase in number only at 48 h postchallenge.

DISCUSSION

By the observation of epidermal sheets stained with anti-Ia antibody, we have previously shown that in allergic contact sensitivity reactions of mice, Ia antigen expression on the keratinocyte surface is recognized from 3–9 days postchallenge [12]. In that study, however, quantitative analysis was not done, since it was impossible to determine how many epidermal cells were expressing Ia antigen. The results of the present study showed that 15–19% of epidermal cells become Ia antigen-positive 48–120 h postchallenge. If we subtract 2% (the percentage of Ia antigen-positive cells in the control epidermis that is composed of only Langerhans cells), it means that about 13–17% of epidermal cells come to newly express Ia antigen. These Ia^+ cells seem to be keratinocytes, because in the observation of the epidermal sheets [12] we recognized that the number of Langerhans cells showed little change in the course of the challenge phase of contact sensitivity. Furthermore, Ia antigen on the keratinocyte surface actually begins to appear 48 h after challenge, 24 h earlier than it is recognizable by the observation of epidermal sheets under the fluorescence microscope. We think that this time discrepancy is due to the difference of the sensitivity of the methods used in the analysis.

Ia antigen expression on keratinocytes has been reported to be induced by gamma-interferon in vitro [18]. Gamma-interferon is produced by sensitized T lymphocytes challenged with the antigen [19,20]. Thus in the delayed contact hypersensitivity reactions of mice, it is reasonable to believe that the Ia antigen expression on the keratinocytes is induced by gamma-interferon secreted by infiltrating T lymphocytes.

The cytofluorography of normal epidermal cells stained with anti-Thy-1 antibody shows 2 peaks, i.e., a broad peak with weak fluorescence intensity and a small peak with strong intensity. Scheid et al [21] demonstrated that a majority of epidermal cells express Thy-1 antigen by cytotoxicity studies. The fluorescence antibody study also showed that, other than strongly Thy-1 an-

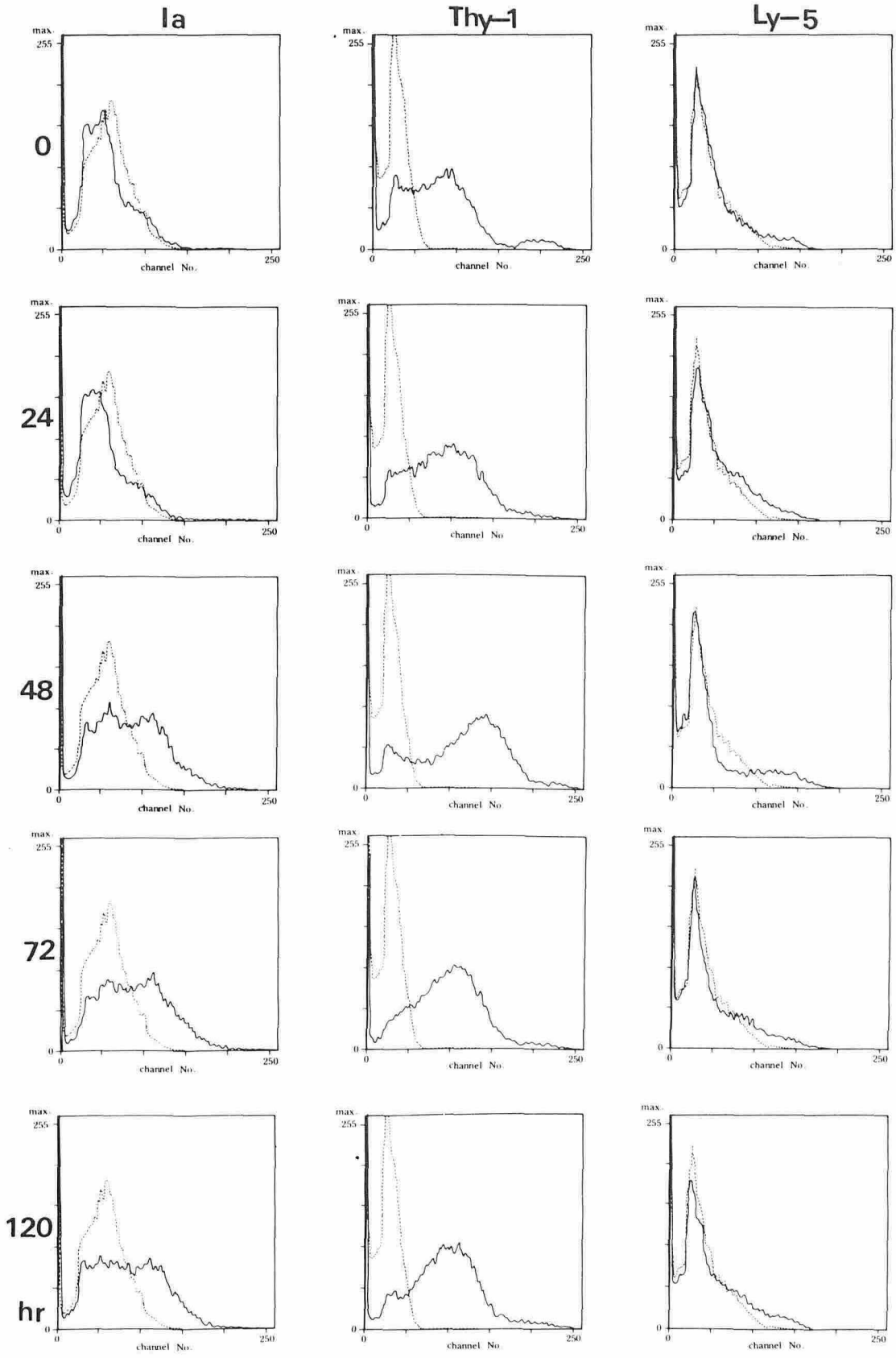

Figure 2. Cytofluorographs of epidermal cells at various times after challenge. The epidermal cells prepared from the ears at various times after challenge were stained with anti-Ia or anti-Ly-5 antibodies and $F(ab')_2$ fraction of FITC-conjugated antimouse immunoglobulins step by step or with FITC-conjugated anti-Thy-1 antibody. 10,000 cells per assay were analyzed by passage through a FACS analyzer. Cytofluorographs of the cells stained with anti-Ia^k, anti-Thy-1, or anti-Ly-5 antibody (——) compared with those of the cells reacted with control unreactive monoclonal antibody (– – –) are shown for each sample. Fluorescence intensity (log units) is expressed on the X-axis and cell number on the Y-axis.

Table I. Flow Cytometric Analysis of Epidermal Cells Reactive with 3 Monoclonal Antibodies After TNCB Challenge

Time After Challenge (h)	Cell Count[a] ($\times 10^{-7}$)	Positive Cells (%) Ia	Thy-1 (high intensity[b])	Ly-5
Control	2.1	2.0	50.1 (5.0)	3.5
0	2.1	2.2	51.8 (5.1)	3.9
12	2.1	2.9	59.7 (2.4)	3.5
24	2.1	3.6	64.4 (3.7)	3.9
48	2.1	15.8	72.1 (11.0)	8.7
72	2.1	17.8	70.9 (3.7)	5.8
120	2.3	19.4	70.6 (4.2)	5.9

[a]The number of cells obtained from both ears of 5 mice at different times after challenge.

[b]The percentage of cells with high fluorescence intensity above the 170 channel in relative fluorescence intensity.

tigen-positive cells, approximately 20–30% of keratinocytes expressed a small amount of Thy-1 antigen [13]. Therefore, we think that the broad peak with weak intensity corresponds to the Thy-1$^+$ keratinocytes, while the small peak with strong intensity corresponds to dendritic Thy-1$^+$ epidermal cells which have been reported by Tschachler et al [13] and Bergstresser et al [14]. We found that about 45% of normal epidermal cells are weakly Thy-1 antigen-positive. This discrepancy of the number of Thy-1$^+$ cells between our study and that of Tschachler et al [13], who used immunofluorescence microscopy, probably resulted from the difference in methodology.

Thy-1$^+$ cells increased in number gradually after challenge to reach a plateau at 48 h postchallenge. Among them, however, the number of strongly Thy-1 antigen-positive cells showed only a small increase of 11% at 48 h postchallenge. Thus it is Thy-1$^+$ keratinocytes that increased in number after challenge. However the determination of the subpopulation of keratinocytes expressing Thy-1 antigen as well as the meaning of their increase in contact sensitivity reactions awaits further investigation.

As compared with Thy-1 antigen which is expressed on both the keratinocytes and dendritic Thy-1$^+$ epidermal cells, Ly-5 antigen and asialo-GM_1 are very specific to the dendritic Thy-1$^+$ epidermal cells in the epidermal cells [13,15]. Therefore it is reasonable to think that in flow cytometry the percentage of Ly-5$^+$ cells in the epidermal cells shows the precise number of dendritic Thy-1$^+$ epidermal cells, although we cannot totally rule out the contamination of T lymphocytes infiltrating into the epidermis. The percentage of Ly-5$^+$ cells did not show any change until 48 h postchallenge, when their number showed an increase, corresponding to that of the strongly Thy-1 antigen-positive cells. However, whether the increase in Ly-5$^+$ and strongly Thy-1 antigen-positive cells at 48 h postchallenge is solely due to an increase in dendritic Thy-1$^+$ epidermal cells remains to be elucidated by further cellular characterization using double labeling with anti-asialo-GM_1, anti-Lyt-1, and anti-Lyt-2.

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